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Award Number: W81XWH-08-1-0447

TITLE:
Respiratory Challenges in Breast Cancer: Potential for Enhanced Diagnostics and Therapy

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REPORT DATE: July 2010

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
504 Scott Street, Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:
☑ Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
31-07-2010	Annual	(1 JUL 2009-30 JUN 2010

4. TITLE AND SUBTITLE
Respiratory Challenges in Breast Cancer: Potential for Enhanced Diagnostics and Therapy

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-08-1-0447

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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8. PERFORMING ORGANIZATION REPORT

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
For the second year of this project, we continued to monitor the changes in endogenous tissue chromophores such as oxy-, deoxy-, total hemoglobin concentration, tissue oxygen saturation, lipid and water contents during tumor growth and chemotherapy using a diffuse optical imaging system. From clinical study, we found that responding tumors show oxyhemoglobin flare within 1 week post neoadjuvant chemotherapy. To confirm this, we monitored the changes of endogenous chromophores post chemotherapy and compared those with controls. We found that functional changes such as oxy-, deoxyhemoglobin, and water precedes the change in tumor volume. This proved our hypothesis that functional changes should occur prior to physical changes when tumors respond to therapy. We have also administered vascular disrupting agents and monitored the changes in tumor oxygenation and blood volume and compared those with contralateral normal breast tissues. We found that vascular disrupting agents work mainly on tumors and also they are more effective in large tumors than small tumors. We also employed Raman spectroscopic measurements on normal tissues near tumor cell inoculation and observed the Raman signal changes as tumor grows.

15. SUBJECT TERMS
Diffuse Optical Imaging, Tumor Angiogenesis, Chemotherapy Monitoring, Breast Cancer, Hyperoxic Gas Intervention

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON: USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE	UU	28	19b. TELEPHONE NUMBER *(include area code)*
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2009-2010 ANNUAL SUMMARY REPORT (YEAR 2)

This report presents the specific aims and accomplishments of our breast cancer research project during the second year of funding sponsored by the U.S. Army Department of the Defense. It covers our activities from July 1, 2009 to June 30, 2010.

INTRODUCTION

Since tumor vasculatures and metabolism are different from normal tissues (Jain, 2005; Tayek, 1992), tumor will respond to hyperoxic/hypoxic gas intervention differently. Hyperoxic gas intervention will visualize tumors from non tumors by enhancing oxygenation in tumors better than non tumors. Compare to currently available exogenous contrast materials used for MRI, PET, and Fluorescence Imaging, this hyperoxic/hypoxic gas intervention is cost effective and safe to use for human which can be easily applied to clinical trials. Tumor oxygenation also plays a key role in cancer treatment. Higher oxygenation in tumor improves the efficacy of radiation therapy (Gray *et al.*, 1953), photodynamic therapy (Henderson and Fingar, 1987), and some type of chemotherapy (Brown, 1993; Teicher *et al.*, 1981; Vaupel, 2002). Meanwhile, it has been found that other type of chemotherapeutic drugs responds better when the tumor is in hypoxic condition (Brown, 1993; Siemann *et al.*, 1991). Therefore, modulating tumor oxygenation could be used to enhance the treatment efficacy depending on the type of therapy. The goals of this research are to enhance breast cancer detection, to improve chemotherapy efficacy, and to predict tumor responses to treatments by incorporating hyperoxic/hypoxic gas interventions into non invasive optical imaging system that we have developed.

BODY

The following tasks have been proposed in our approved statement of work.

Task 3: To observe the respiratory intervention effects on tumor blood flow using a skin fold window chamber model using a laser speckle imaging (LSI) system. **(months 13-14)**:

a. To surgically install a window chamber on Fisher 344 rats (months 13-14)
b. To implant breast tumors in the window chamber. (months 13-14).
c. To perform LSI measurements on tumors in window chamber to observe blood flow changes during hypercapnic/hyperoxic/hypoxic gas interventions. (months 13-14).

Task 4: To monitor tumor responses to chemotherapy with or without respiratory interventions. **(months 15-24)**:

a. To breed brca1/p53 transgenic mice (n=328) for this task. (months 9-10)
b. To write an animal protocol and get an approval. (months 13-14)
c. To perform MI/LSI measurements on breast tumors (n=24) with Tirapazamine treatment with/without hypercapnic and hypoxic gas interventions during treatment. (months 15-18)
d. To perform MI/LSI measurements on breast tumors (n=24) with Cisplatin treatment with/without hypercapnic and hyperoxic gas interventions during treatment. (months 19-22)
e. To perform histology/immunohistochemistry on harvested tumors. (months 15-23)
f. To compare the tissue optical composition changes/hemodynamic changes during chemotherapy with histology/immunohistochemical results. (months 24)

It is very unfortunate that we could not accomplish our second year tasks. Our proposed model in task 3 was a dorsal skinfold window chamber model which inoculates tumor cells in the dorsal skin rather than

in the breast of rats. However, we found that there are great differences in terms of tumor growth, metastasis, and therapy efficacy between ectopic and orthotopic tumor model. Therefore, we tried to adapt a mammary gland window model from Dr. Dewhirst's lab (Shan *et al.*, 2003). However, we failed to reproduce their model in our lab and need to decide whether to follow our original dorsal skinfold window model despite its disadvantages or to have training from Dr. Dewhirst's lab.

We could not complete the proposed work in task 4. We performed a series of animal imaging under cisplatin treatment during our first year of this project. However, the spontaneous Brca1/p53 knockout mice tumor model normally takes 5 to 6 months to have tumors grow on the mice and we couldn't have enough animals to perform all the work in task 4. In addition, we were not able to obtain Tirapazamine drug. Instead of performing Task 3 and 4, we spent our efforts on 1) investigating oxy-, deoxyhemoglobin flares post chemotherapy using two different tumor lines, 2) monitoring and comparing the effects of vascular disrupting agents between small vs. large tumors in our animal model, 3) watching the Raman signal changes at the site far away from tumors. These new studies are summarized in this report.

Accomplishments during this period (months 13-24):

a. We obtained quantitative changes in endogenous chromophores such as oxy-, dexoy-, total hemoglobin, lipid, water, and tissue oxygen saturation from 13762MAT B III mammary tumors on Fisher 344 rats during its growth and also during chemotherapy using cyclophosphamide.

b. We compared the effects of cyclophosphamide treatment on endogenous chromophores between small and large tumors.

c. We compared the efficacy of vascular disrupting agents, combretastatin A4 phosphate and ECA, and also between small and large tumor groups using optical imaging system.

d. We obtained changes of oxy-, dexoy-, total hemoglobin, lipid, water, and tissue oxygen saturation from slow growing tumor, R3230AC mammary tumors on Fisher 344 rats during its growth and also during chemotherapy using cyclophosphamide. We also compared endogenous signals changes post cyclophosphamide treatment between R3230AC and 13762MAT B III tumors.

e. We acquired Raman spectra from tumors and normal tissues, and monitored Raman signal change at normal tissue region as tumors grow.

1. Endogenous Optical Signals Changes in Rat Mammary Breast Tumor Post Chemotherapy

Once animals had enough time to adapt themselves in a new environment after their arrival (> 3days), we divided animals into two groups. A treatment group was administered with a high single dose (100mg/kg) of cyclophosphamide i. p. when tumors became ~1cm diameter while control group received an inactive vehicle. We inoculated a half million of 13762 MAT-III tumor cells in the right caudal mammary fat pad of female Fisher 344 rats (170~190g) for the treatment group. Control group had one million cells inoculation to expedite tumor growth. We acquired diffuse optical images daily from the day before tumor cell inoculation until sacrifice using modulated imaging (MI) system. MI system acquired the raw images from the breast tissue surface by projecting the two spatial frequency patterns of NIR light with wavelengths from 650 to 990 nm at every 20nm. From the raw images, the quantification of oxy- ([OHb]), deoxy- ([RHb]), total [THb] hemoglobin, tissue oxygen saturation (S_tO_2(%)=[OHb]/[THb] X 100), lipid, and water contents as well as the light scattering were carried out.

Control animals lost their body weight slightly mean while treatment group showed minimum body weight 4-5 days post cyclophosphamide administration then gradually gained weight as tumors regress their size. Tumor volume was estimated with ellipsoidal volume approximation. (Tumor volume = 4/3*π*a*b*c where *a* and *b* are the equatorial radii (along the *x* and *y* axes) and *c* is the polar radius

(along the z-axis)) Control animals showed continued growth before sacrifice and treatment group showed its maximum volume at 2 days post cyclophosphamide administration followed by a gradual regression showing the response to chemotherapy. (Figure 1)

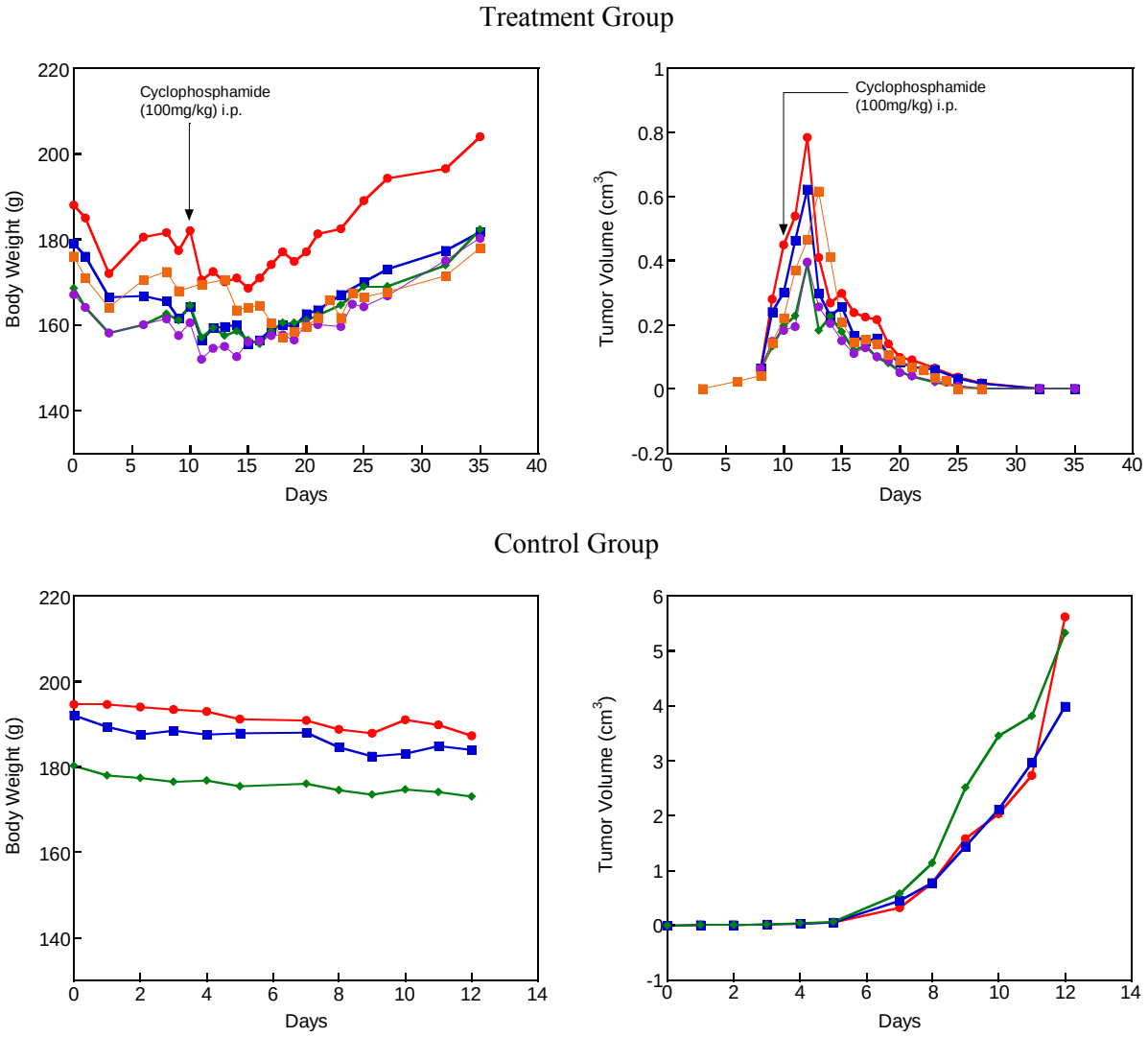

Fig. 1 Body weight (top left) and tumor volume changes (top right) from chemotherapy treatment group and control group (bottom left and right).

Figure 2 shows the representative endogenous chromophores concentration changes (lines without symbols) and tumor volume changes (purple dotted line with circles) from tumor and normal breast in control group. Tumor breast showed increases of OHb and THb until day 9 followed by a regression. However, RHb and water continued to increase, and therefore, StO2 values decreased at day 10 to 12. These results show that most signals detected by MI system are from necrotic region in tumors starting from day 10. Lipid content dropped as tumor grows and was undetectable at day 6. We expected to have stable values of endogenous chromophores concentrations from control breast, but they also showed small but gradual increases of OHb, RHb and THb. This may be explained by a recruitment of blood vessels surrounding tumor region as tumor grows which will be detected even at normal breast region since the distance between control and tumor breast became less than1 cm at day 12.

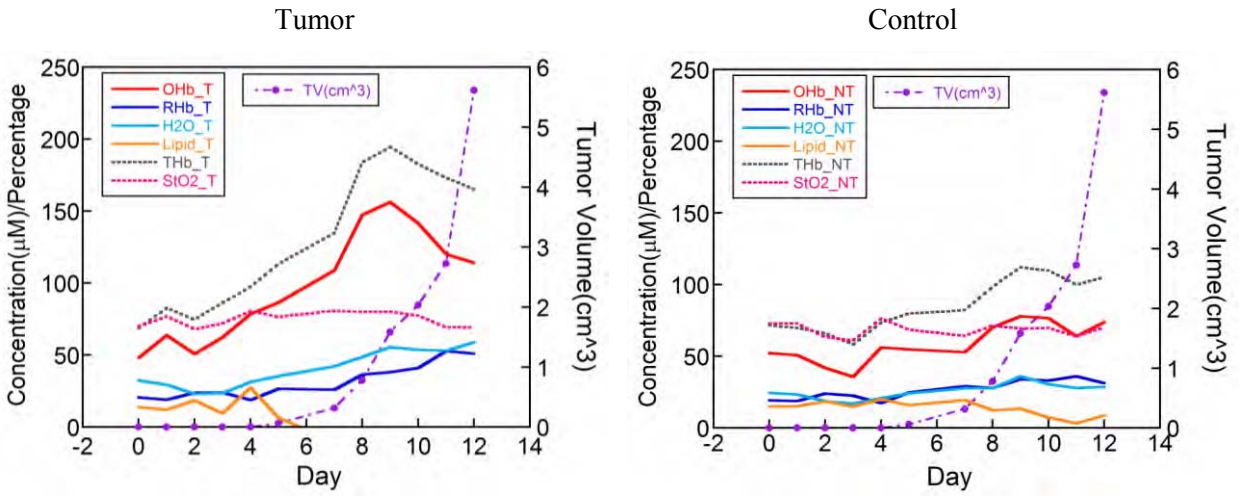

Fig. 2 Endogenous chromophores concentration changes from control group. The left is from tumor breast and the right is from contralateral normal breast.

Figure 3 shows a representative changes of OHb, RHb, Lipid, and Water from cyclophosphamide (CTX) treatment group. OHb, RHb, and water increase as tumor grows while lipid gradually decrease. After a single dose of cyclophosphamide treatment, OHb and RHb showed their maximum at 1 day post chemotherapy then decreased. The tumor continued to grow even after CTX treatment and reached its maxium volume at 2 days post chemotherpay then regressed its size. Right panel of Fig. 3 shows the amplitude changes of OHb, RHb, THb, and StO2 during oxygen intervention from the same animal. As the tumor grows Δ[OHb], Δ [RHb], Δ[StO2] also increased. However, those values were dropped greatly right after the chemotherapy and slowly decreased. Interestingly, Δ[OHb] jumped at 3 day post treatment which may be related to the change of tumor vascularization after chemotherapy.

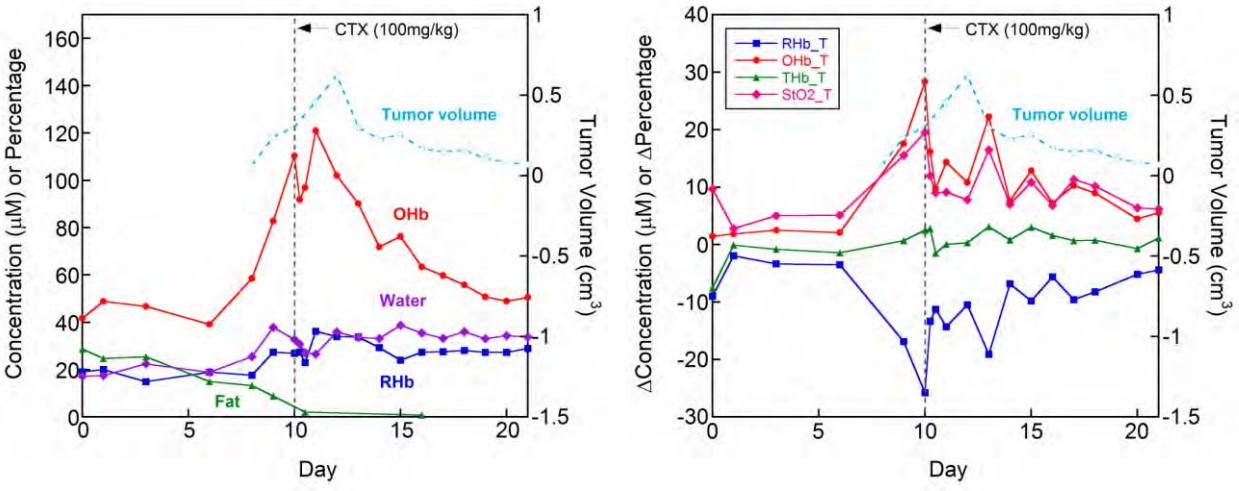

Fig. 3 The changes of OHb, RHb, lipid, and water from the tumor treated with a single dose of cyclophosphamide treatment (left) and the changes during oxygen intervention of OHb, RHb, THb, and StO2 were plotted on the right panel.

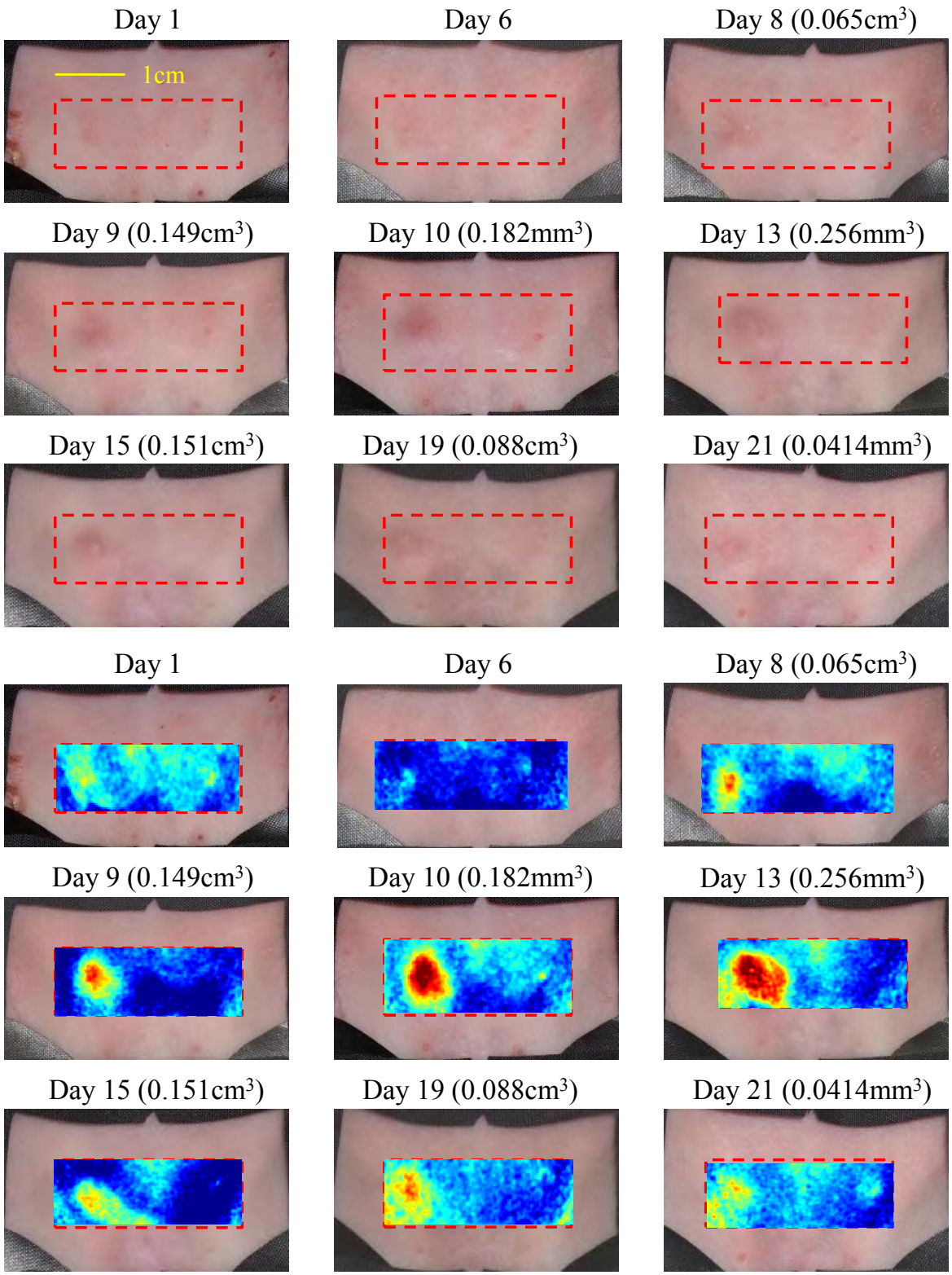

Fig. 4 (top) the digital color photos of the tumor during its growth and post chemotherapy (at day 10) and (bottom) scattering b values are overlaid on color photos.

These endogenous chromophore concentration changes were calculated from absorption spectra and we also looked the changes in scattering values from the same animal shown in Fig. 3. In the near infrared region (650-1000nm), scattering values at each wavelength can be fitted with a power law ($\mu_s' = A \cdot \lambda^{-b}$). Figure 4 shows the color photos during the tumor growth and also regression post chemotherapy on the top and scattering b value maps overlaid on the color photos at the bottom. This may show an implication of using scattering map to detect tumors.

2. Flares Observed from Rat Mammary Breast Tumor Post Chemotherapy

From our clinical study, we found that complete and partial response tumors show an oxyhemoglobin flare within 1 week post chemotherapy while non responding tumors did not show a flare as shown in Fig. 5. When we plot the maximum oxyhemoglobin change during the 1st week post chemotherapy, we could differentiate between CR/PR and NR groups. This encouraged us to test on our animal model which was described in the previous section.

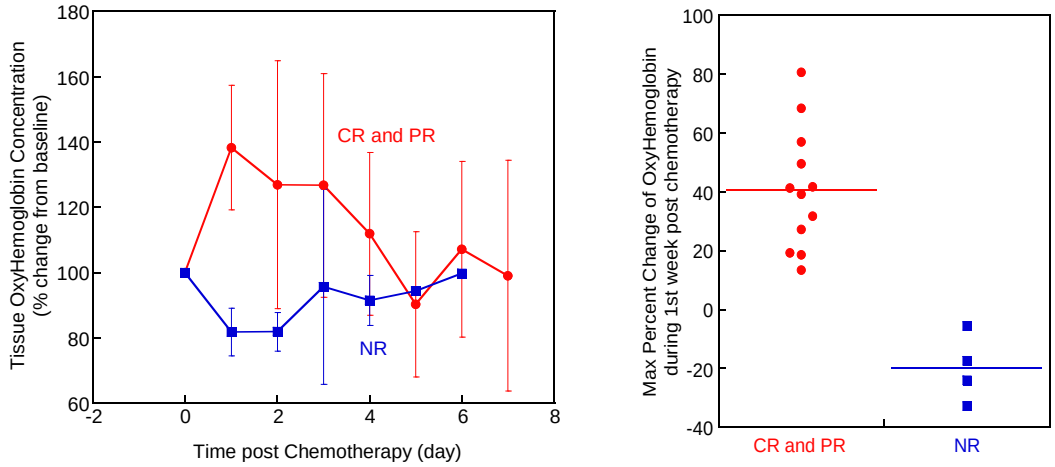

Fig. 5 (Left) the percentage change of oxyhemoglobin from complete response (CR), partial response (PR), and non response (NR) groups post neoadjuvant chemotherapy. (Right) a scatter plot showing the maximum change of oxyhemoglobin during the 1st week post chemotherapy between CR/PR and NR groups.

Figure 6 shows the results from animal tumor study. Each chromophore concentration changes were normalized to the values from right before the cyclophosphamide treatment. Normal breast showed increases of OHb, RHb, and THb right after administration of CTX and returned to the values at day 0 post chemotherapy. Tumors showed an initial drop of OHb, THb, and water followed by flares within 2 days post treatment. RHb continued to increase right after treatment also reached to its maximum value at day 1 post treatment. However, water percentage was initially dropped at 6, 12 and 24 hrs from the baseline values then showed its maximum at day 2 post CTX treatment. Tumors continued to grow even after the treatment and reached to maximum size at day 2 post chemotherapy.

The tumors shown in Fig. 6 were having a volume in the range from 0.2 to 0.5 cm^3 when the chemotherapy was applied and all showed a complete response. We then applied chemotherapy to the other group of animals (n=5) when tumors were quite large (3~8 cm^3) and monitored the change of endogenous signals at 0, 6, 12, 24, 48 hrs post treatment (Fig. 7). Unlike small tumors' response, these large tumors did not show the oxyhemoglobin flare. Two of animals were sacrificed at 24hrs post treatment and three animals were sacrificed at 48 hrs post treatment to perform immunohistochemical analysis using Ki67 and CD31 (Fig. 8).

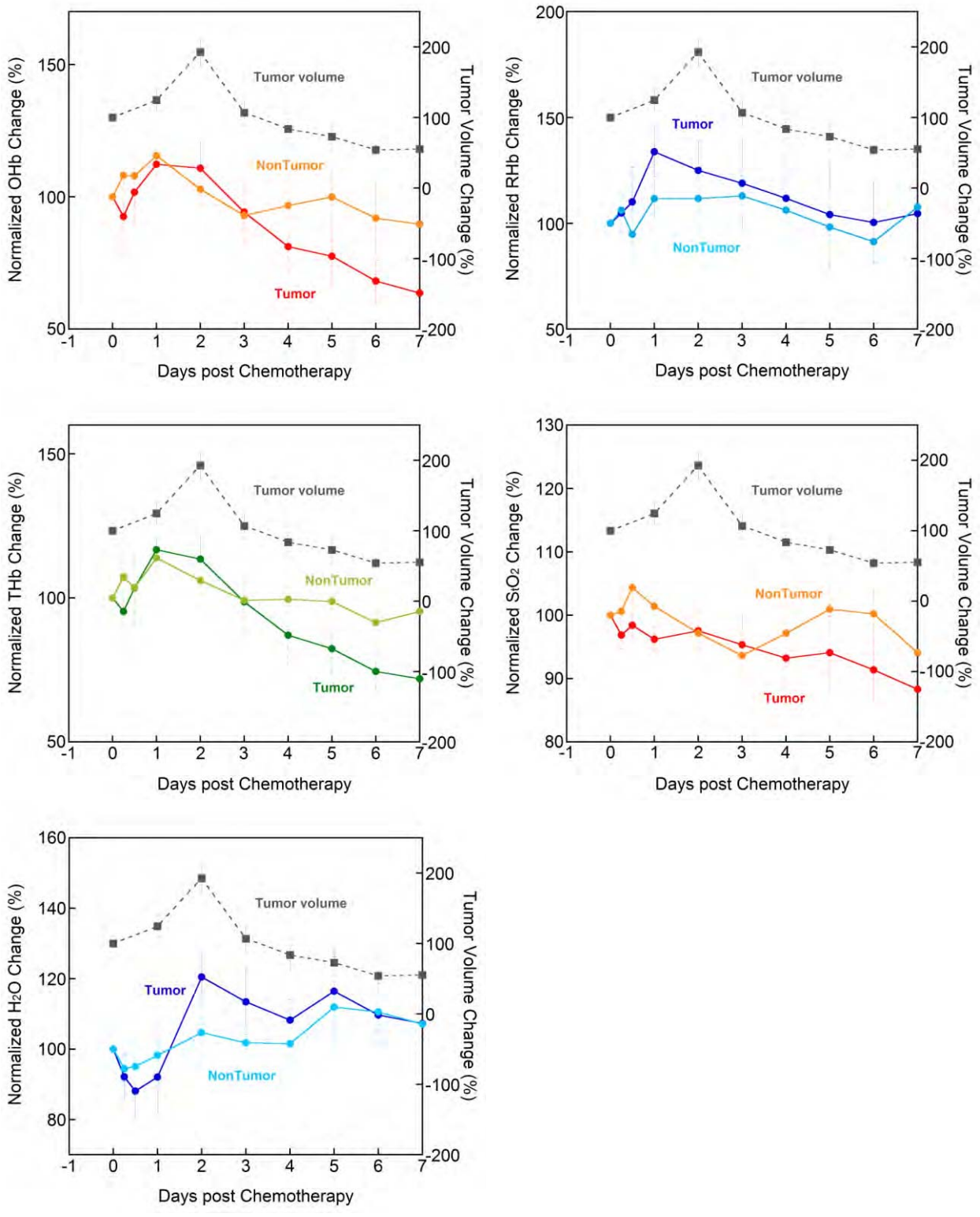

Fig. 6 The averaged percentage changes of OHb, RHb, THb, StO2, and water from small tumors and normal breast after a single high dose (100mg/kg) of cyclophosphamide treatment. Data were obtained at 0, 6, 12 hr, 1 to 7 days post treatment.

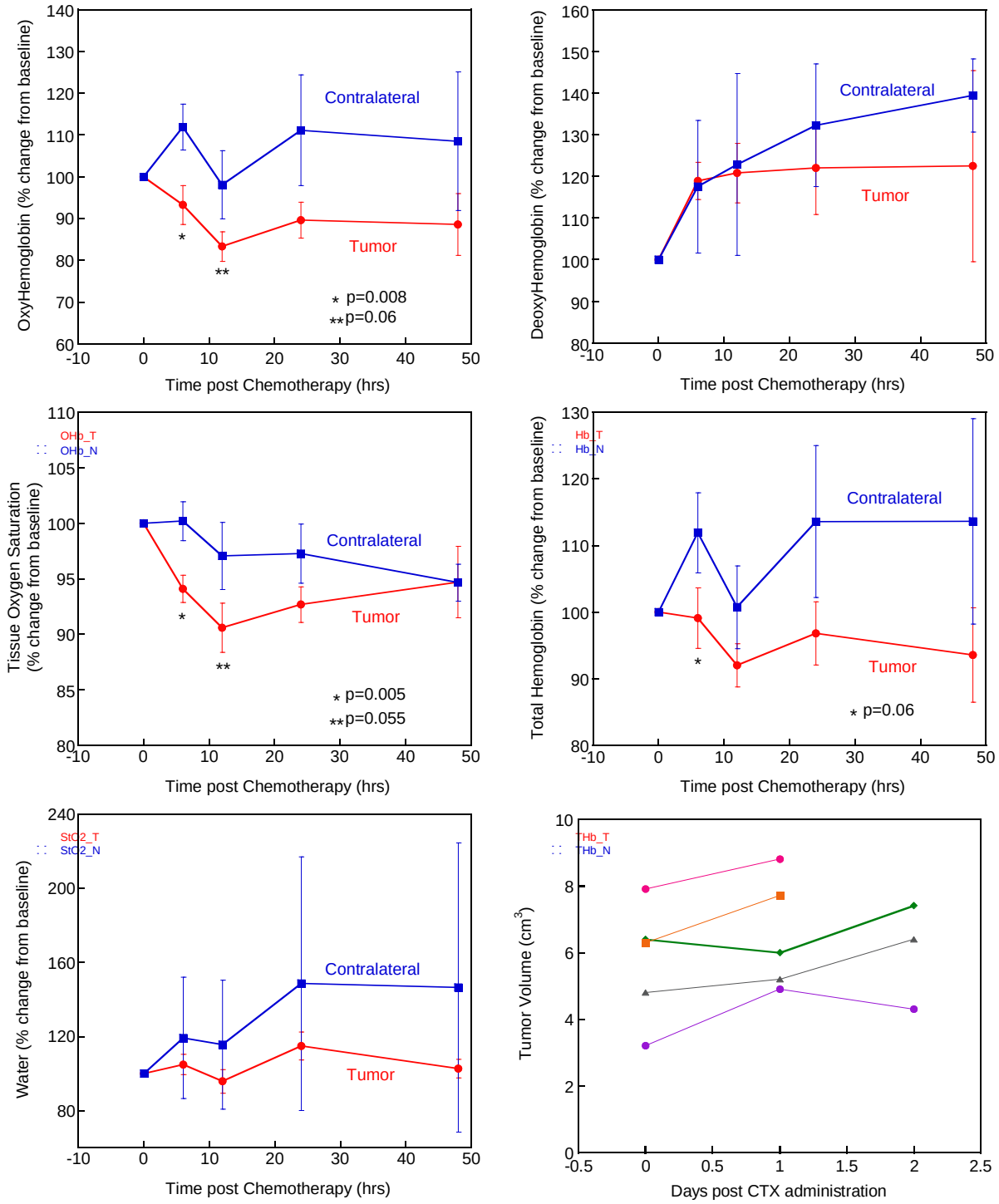

Fig. 7 The averaged percentage changes of OHb, RHb, THb, StO2, and water from large tumors and normal breast after a single high dose (100mg/kg) of cyclophosphamide treatment. Data were obtained at 0, 6, 12, 24, 48 hrs post treatment. Tumor volume changes are shown at the right bottom.

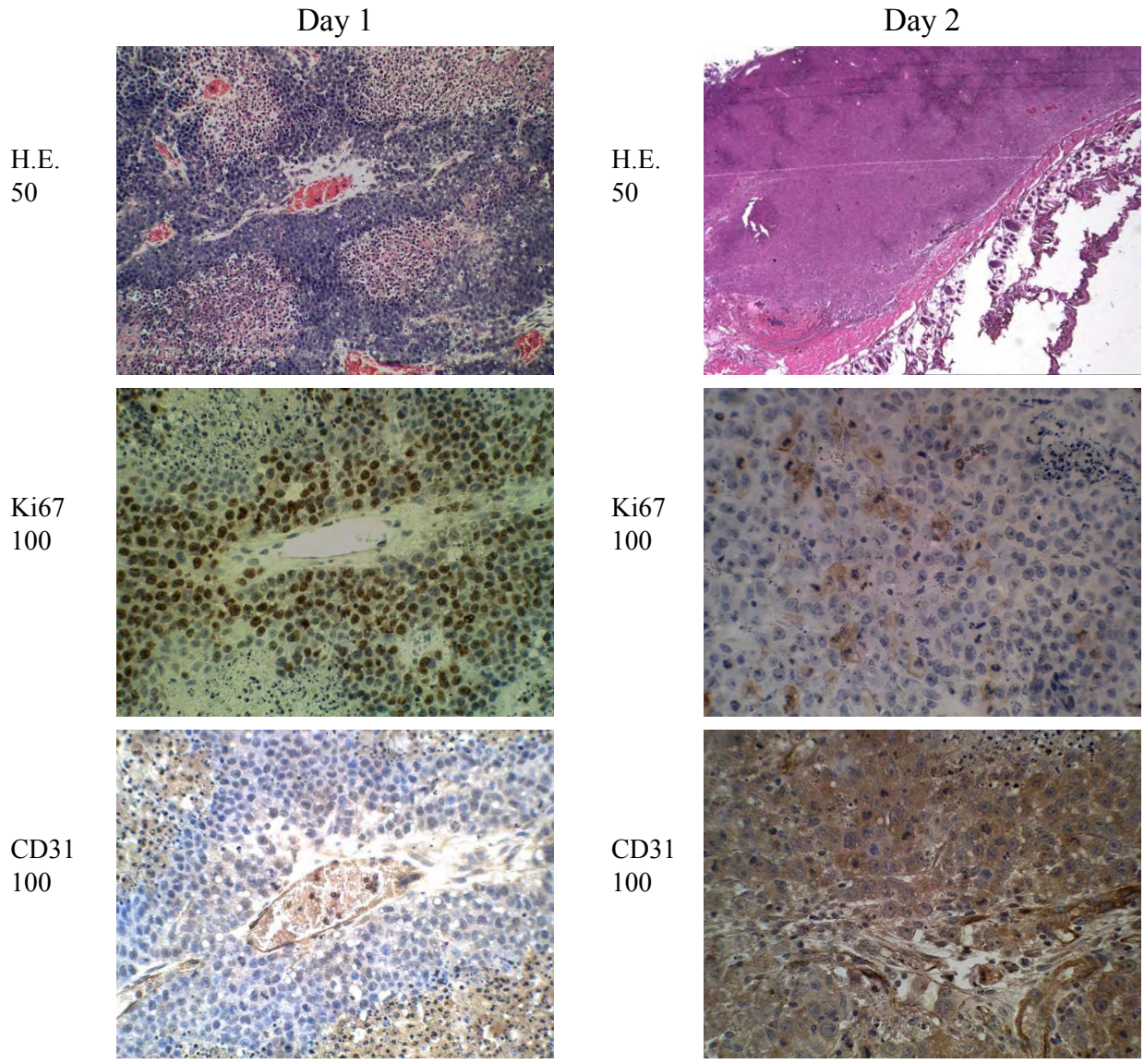

Fig. 8 The immunohistology photos from large tumors post chemotherapy. Left photos are from day 1 post treatment and photos on the right panel are from day 2 post chemotherapy. H&E staining is shown on the top, Ki67 immunostained images are shown in the middle, and CD31 stained images are shown at the bottom.

Ki67 biomarker stains cells that are in active for proliferation and is being used as a prognostic factor to stratify breast cancer patients (Scholzen and Gerdes, 2000; Brown and Gatter, 1990). CD31 is a biomarker that stains the endothelial cells of tumor blood vessels (Albelda *et al.*, 1991). We counted stained cells from Ki67 and CD31 staining images and compared the numbers between day 1 and day 2 post chemotherapy from large tumors (Fig. 9). The results show that the numbers of both Ki67 and CD31 stained cells are decreased from day 1 to day 2 ($p < 0.05$). These implies that cell proliferation is reduced at day 2 compared to day 1 post chemotherapy and the number of blood vessels is also reduced at day 2 from day 1 post treatment.

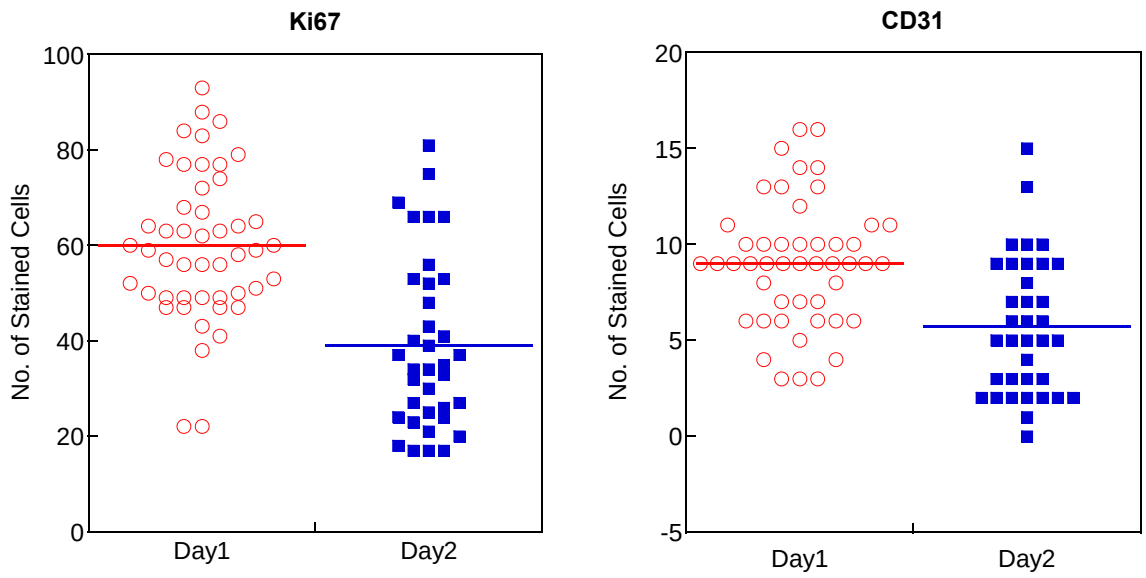

Fig. 9 Scatter plots of number of Ki67 (left) and CD31 (right) stained cells counted at 100X for Ki67 and 20X for CD31. We see the decrease of both Ki67 and CD31 activity from day 1 to day 2.

We also plotted the change of each chromophores vs. tumor volume change (Fig. 10) We can see an increase of all chromophores during the initial tumor growth up to $1cm^3$. However, OHb decreases when tumor grows bigger than $1cm^3$ while RHb continues to rise. This causes a decrease of StO2 which may represent an increase of necrotic tissue within the tumor. Water content also showed an initial rapid increase as tumor grows then followed by a gradual increase which may be associated with edema or necrosis of tumor tissues.

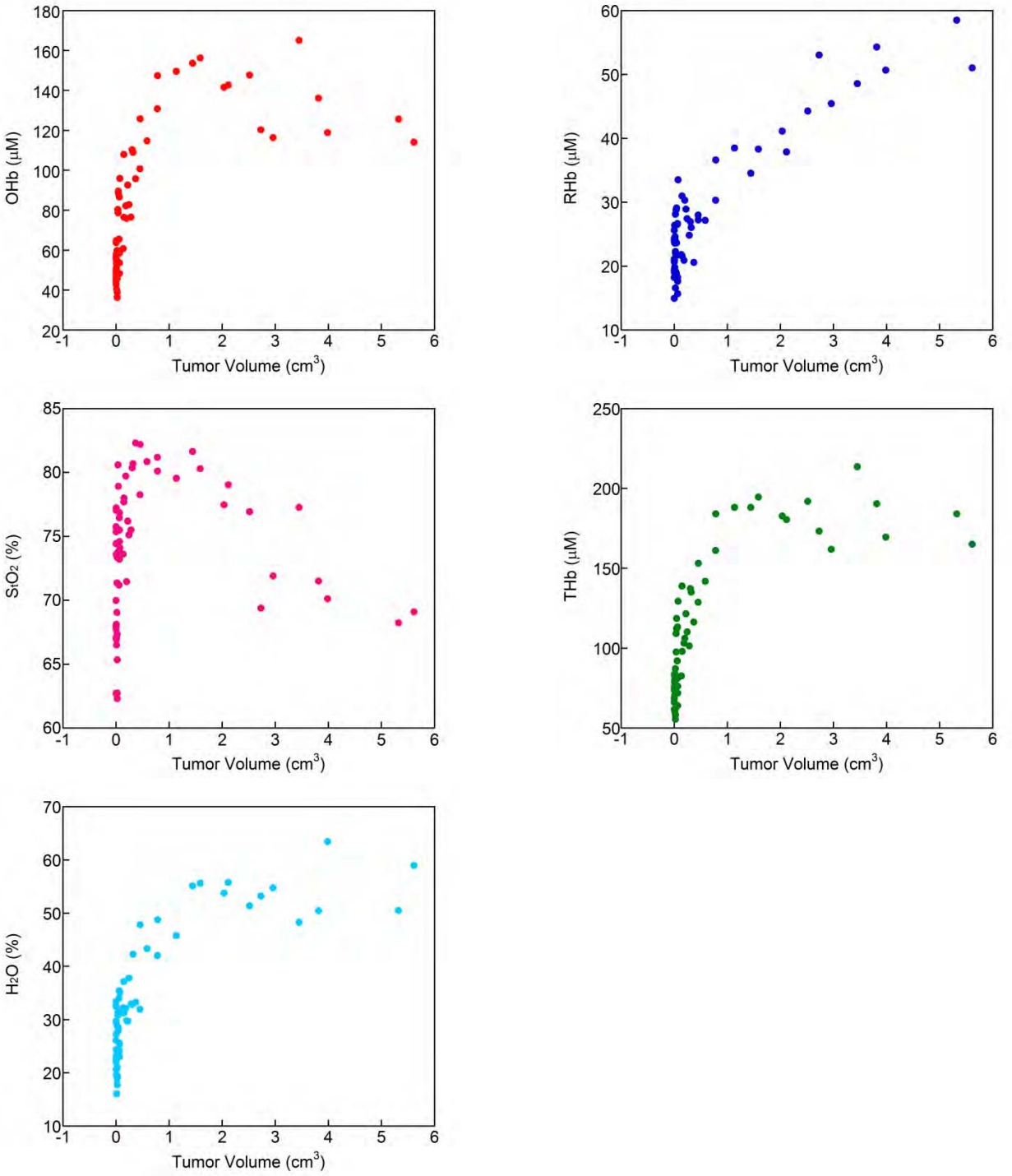

Fig. 10 The change of oxy- (OHb), deoxy- (RHb), total hemoglobin (THb), StO2, and water (H2O) vs. tumor volume change.

3. The Hemodynamic Changes Due to Vascular Disrupting Agents Administration

Vascular disrupting agents such as combretastatin A4 phosphate (CA4P) (Siemann *et al.*, 2009) and 5,6-dimethylxanthenone-4-acetic acid) (DMXAA) (Zhou *et al.*, 2002) have been developed to disrupt tumor

blood vessels and have shown some great results in cancer treatment, especially combined with a conventional chemotherapy (Horsman and Siemann, 2006), photodynamic therapy (Seshadri and Bellnier, 2009) and radiotherapy (Sunar *et al.*, 2007). CA4P is a tubulin binding agent and it depolymerizes tubulin in endothelial cells of tumor blood vessels which results in change of endothelial cell shape from flat to round. This shape change of endothelial cells causes a reduced blood flow, an increase of permeability, and an increase of tumor interstitial fluid pressure that eventually causes tumor cell death. We have tested a newly developed drug, ECA, and compared its efficacy with CA4P in terms of tumor oxygenation and blood volume changes using the same animal model that was described earlier. The dose for both drugs was 30mg/kg and administered via i.p.

Since CA4P is known to work better for the large tumors, we divided animals into two groups and administered VDAs when tumors are small (<0.25cm^3) and when they are large (>1.4cm^3). Figure 11 shows the percentage change of tumor volume from two groups. We can see that small tumors became slow in terms of growth rate after CA4P or ECA treatment while large tumors reduced their size at day 1 post CA4P treatment. However, ECA treatment seems not to affect tumor growth when tumors are large.

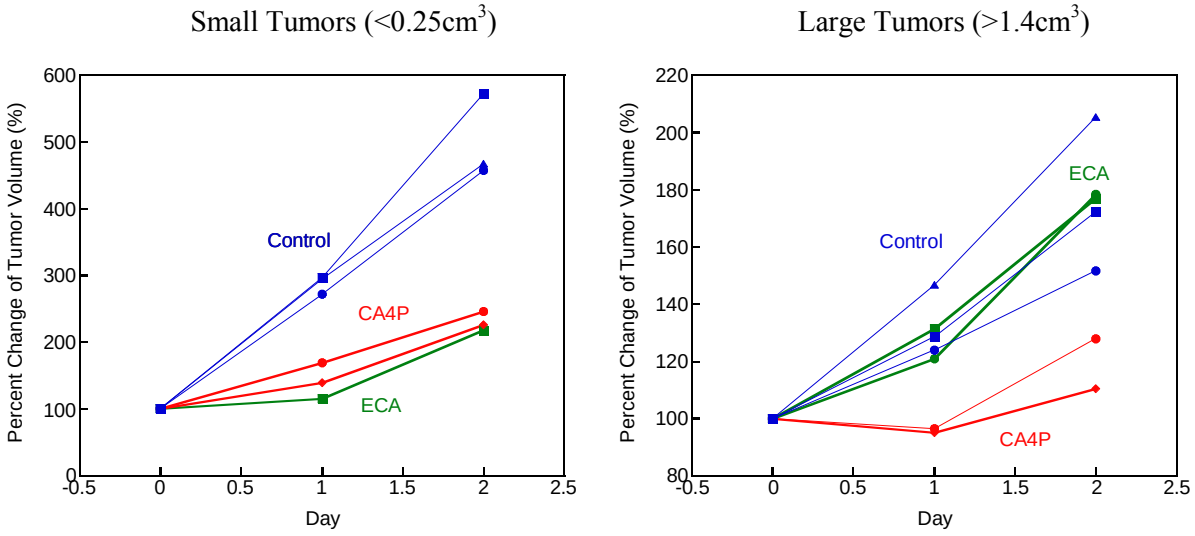

Fig. 11 The percentage change of tumor volume post treatment. Small tumor volume group data are shown on the left and large tumor volume group data are shown on the right.

Figure 12 shows the percent changes of OHb and RHb from both tumor and normal breast before and post administration of saline, CA4P or ECA. Solid lines are from tumors and dotted lines represent signals from normal breast. Respiratory challenges (from 100% oxygen to air) were applied before and 1hr post administration of agents to see whether there will be a change of vascular reaction during air intervention before and after agent administration. Top two plots shows the change of OHb and RHb from saline administered animals. Switch from oxygen to air caused a drop of OHb and a rise of RHb and these were reversed when air was changed back to oxygen. The amplitude of OHb and RHb changes during air intervention became greater at 1hr post saline administration. We found that the air intervention at 1hr post agent administration caused more drop in arterial oxygen saturation values than those before administration. This is due to long duration of anesthesia to the animals and was found from other groups.

Compared to controls, both CA4P and ECA administration caused a gradual decrease of OHb and increase of RHb from tumors while normal breast did show very slight changes. This proves that CA4P and ECA are mainly working on tumor vasculature. We also found that the second air intervention, which was applied at 1 hr post CA4P or ECA administration caused a relatively small changes of OHb and RHb

compare to those from controls. This implies that CA4P and ECA caused a partial tumor vascular collapse and therefore the vascular reaction during air intervention became less than the baseline.

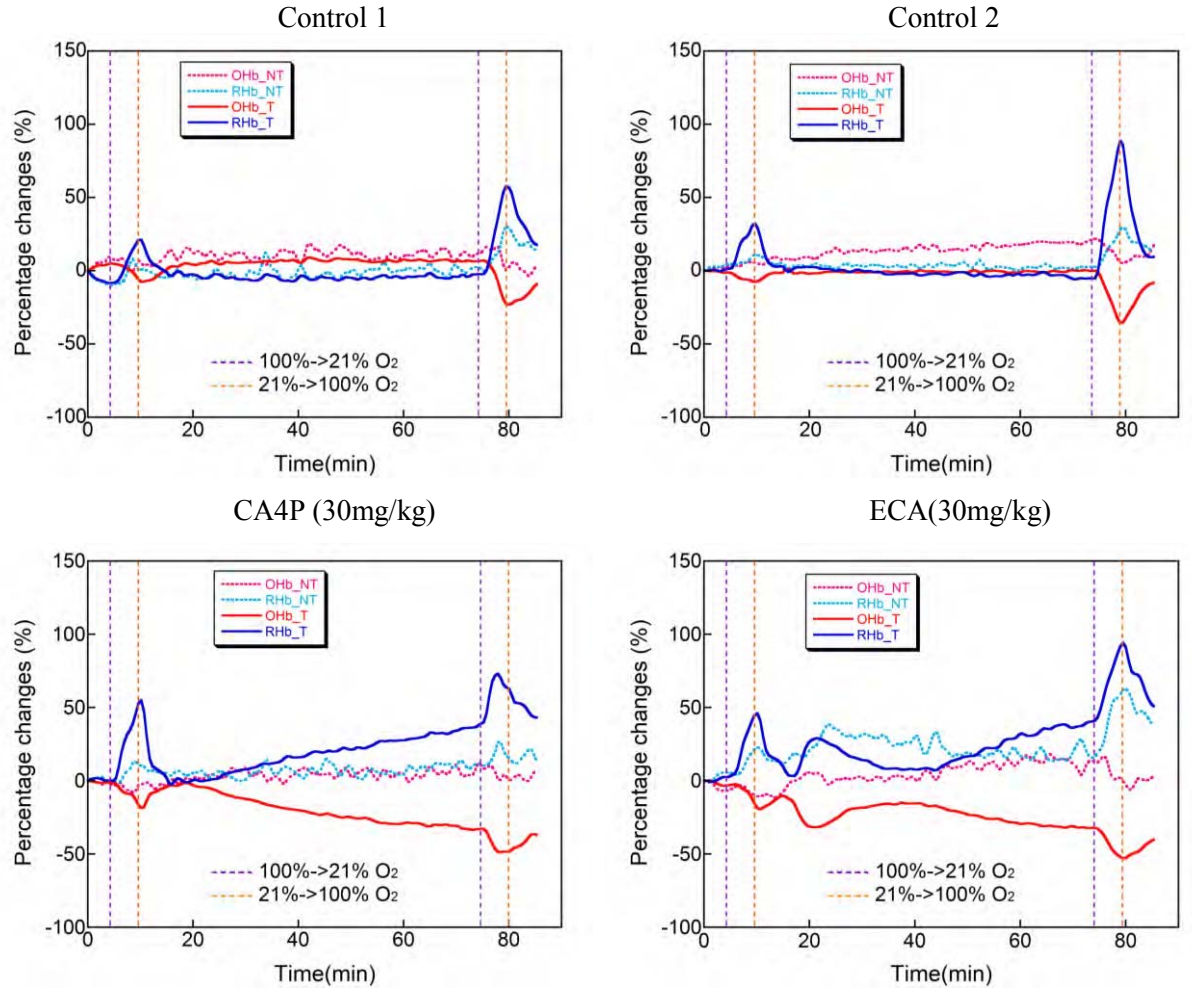

Fig. 12 The changes of OHb and RHb from tumors (solid lines) and contralateral normal breasts (dotted lines). Two control data are shown on the top and CA4P or ECA treatment animal's data are shown at the bottom. Air intervention was applied between a dotted vertical purple line and a dotted vertical orange line.

CA4P and ECA were also administed to other group that has a large tumors and the representative results are shown in Fig. 13. Compared to the data from small tumors, we found greater changes of OHb and RHb post CA4P or ECA treatment from large tumors. This confirmed that CA4P is working better for the large tumors which was also shown by other groups. The amplitudes of OHb and RHb change during air intervention are also much less than those from baseline values.

We also monitored the hemodynamic changes from the tumor that was administered with a high dose (100mg/kg) of cyclophosphamide. Cyclophosphamide is a nitrogen mustard alkylating agent which adds alkyl groups to guanine base of DNA. This cross linking of DNA stops cell growth which kills tumor cells eventually. Unlike CA4P treatment, cyclophosphamide caused decreases of both OHb and RHb in tumors while normal breast showed an increase of OHb post administration, which may be due to an acute inflammation effect from cyclophosphamide treatment. This result clearly demonstrates how different type of chemotherapeutic agent is working by different mechanisms.

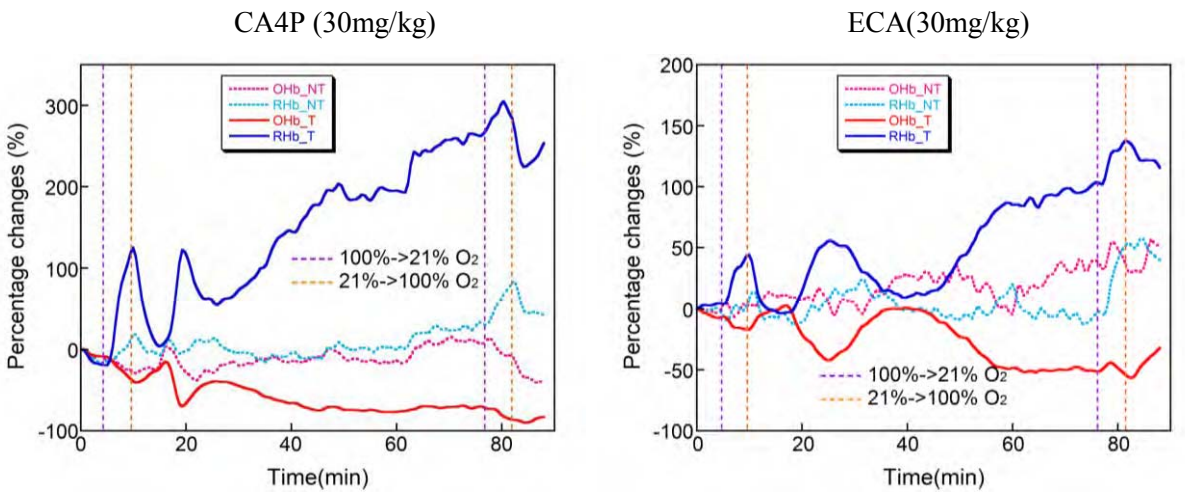

Fig. 13 The changes of OHb and RHb from tumors (solid lines) and contralateral normal breasts (dotted lines). CA4P treated animal's data is shown on the left and ECA treatment animal's data are shown on the right. Air intervention was applied between a dotted vertical purple line and a dotted vertical orange line.

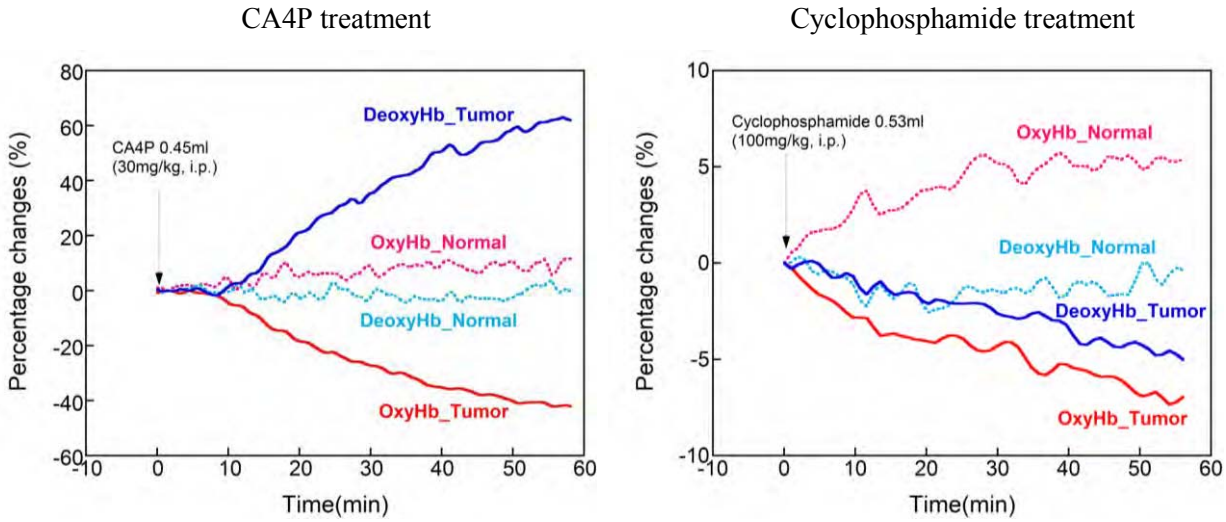

Fig. 14 The changes of OHb and RHb from tumors (solid lines) and contralateral normal breasts (dotted lines). CA4P treated animal's data is shown on the left and ECA treatment animal's data are shown on the right. Air intervention was applied between a dotted vertical purple line and a dotted vertical orange line.

4. Comparison of hemodynamic between two different rat breast tumors post chemotherapy

We showed the changes of endogenous chromophores concentration in tumors post cyclophosphamide treatment at section 2. We found that there are flares of OHb, RHb, and Water at day 1 or 2 after cyclophosphamide administration. However, we had a question of whether these flares are due to chemotherapy or due to the continued tumor growth. To answer this question, we approached in two ways. Firstly, we performed a control study shown in section 2 to see how endogeneous signals change without chemotherapy. Secondly, we applied cyclophosphamide treatment to tumors that grow much slower than 13762 MAT B III tumors. R3230AC tumors were chosen for this study. It is transplantable mammary adenocarcinoma of the Fisher rat, and is spontaneously arisen from the R3230AB which is a faster growing, lactating tumor (Hilf *et al.*, 1965). R3230AC differs from R3230AB by showing a slow growth rate, the predominance of epithelial cell elements, the higher activities of the triphosphopyridine

nucleotide (TPN)-linked dehydrogenase enzymes, and the absence of anaerobic utilization of glucose *in vitro*.

We inoculated 1million of R3230AC cells with the same procedure as 13762MAT B III cells. Figure 15 shows the changes of body weight and tumor volumes from this group. Compared with 13762MAT B III tumors, R3230AC tumors took more than 3 times longer to be the same size as observed from 13762MAT B III tumors. When tumor volume reached at ~1 cm^3, cyclophosphamide was administered i.p. at day 26 with the same dose (100mg/kg) as the one was given to 13762MAT B III tumors. Animals lost their body weight after cyclophosphamide administration as one of the side effects, but regained 1 week post treatment. Unlike 13762MAT B III tumors, R3230AC tumors had a few days of tumor growth delay after cyclophosphamide treatment. However, tumors restarted to grow 1 week after the treatment.

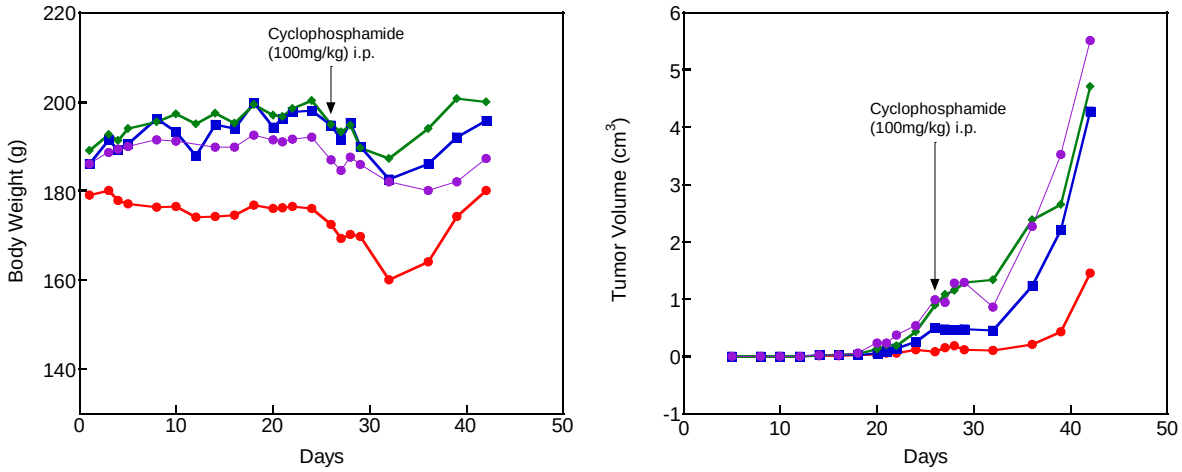

Fig. 15 Body weight (left) and tumor volume changes (right) from R3230AC tumor bearing rats. A single high dose (100mg/kg) of cyclophosphamide was given at day 26.

The endogenous signals changes from tumors and normal breast throughout the whole measurement period are shown in Fig. 16. As tumors grow, OHb and water content increased while RHb decreased. These are the same observation from 13762 MAT B III tumors. After administration of cyclophosphamide, tumor volume slightly rises then went down close to the volume at the day 0 post treatment. Compared to 13762MAT B III tumors, R3230AC tumors showed much slower increase of tumor volume until day 2 post chemotherapy. Normal breast showed elevated levels of OHb, RHb and water post chemotherapy (Fig. 17). However, we did not observe flares of OHb and RHb from this tumor line after cyclophosphamide treatment as compared to 13762 MAT B III tumors shown in Fig. 6. Interestingly, water level was increased from both tumors and normal breast for R3230AC tumor line which may be related to the level of necrosis in tumors.

We sacrificed animals at the end of experiments and collected tumor tissues to see the histological differences between two tumors. As we can see in Fig. 18, both 13762 MAT B III and R3230AC tumors show huge central necrosis. However, the central region of R3230AC tumor tissue is still intact while 13762MAT B III tumors basically lost all tissues. R3230AC tumors also have some fat regions at the side section, which may be due to its lactating characteristics.

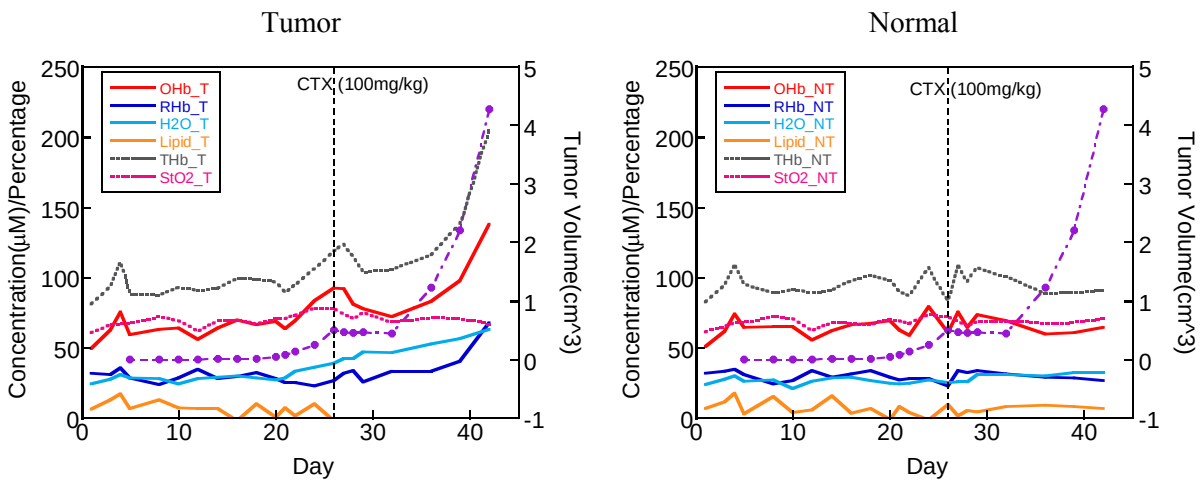

Fig. 16 Endogenous chromophores concentration changes from R3230AC tumor group. The left is from tumor breast and the right is from contralateral normal breast.

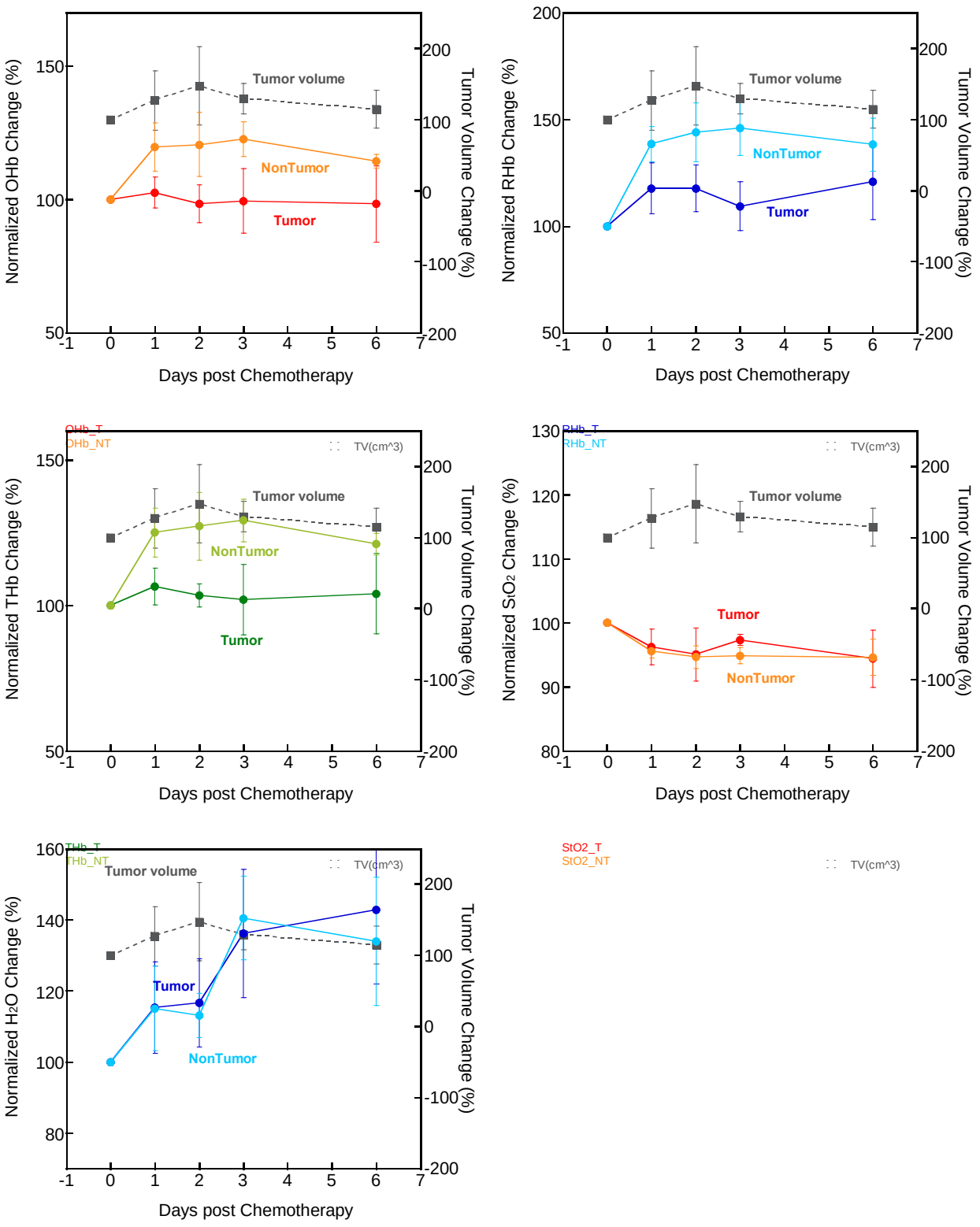

Fig. 17 The averaged percentage changes of OHb, RHb, THb, StO2, and water from R3230AC tumors and contralateral normal breast after a single high dose (100mg/kg) of cyclophosphamide treatment.

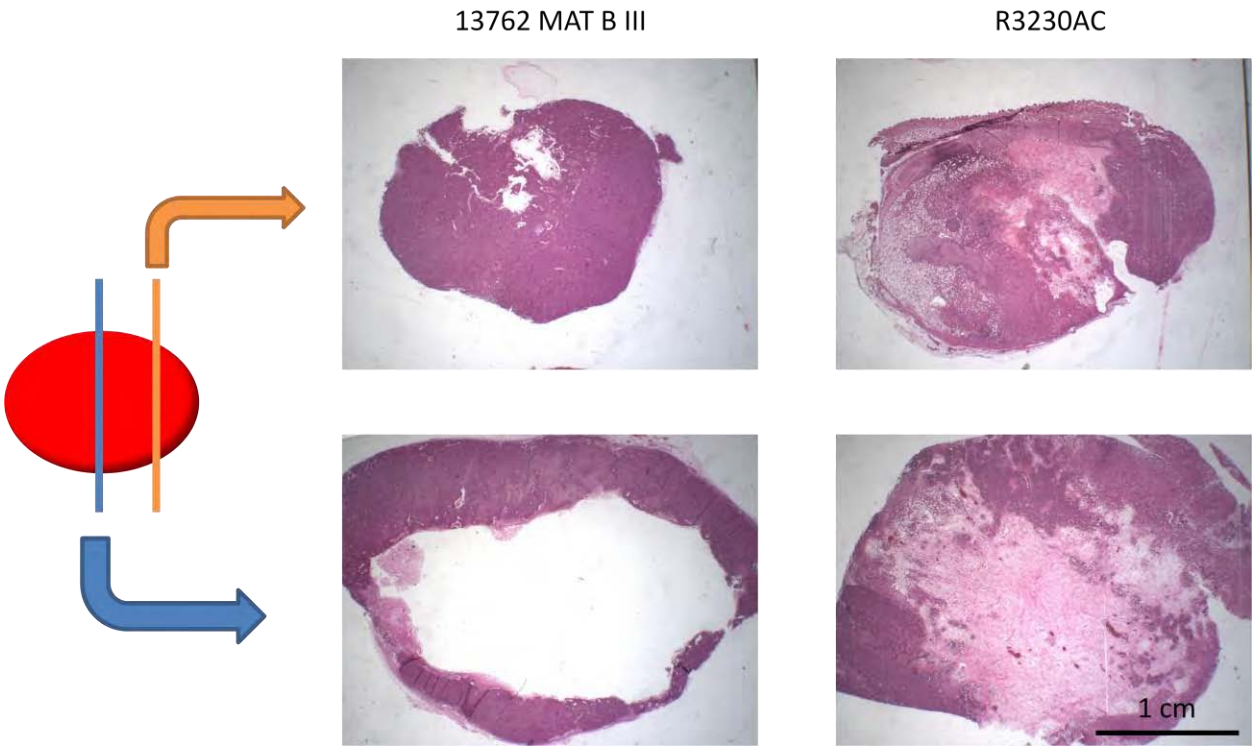

Fig. 18 The H&E stained sections of 13762 MAT B III (left) and R3230AC (right) tumors from two regions. Top sections are close to the side and bottom sections are from center of tumors. Both tumors show huge necrotic area in the center.

5. Raman spectral detection of cancer field effects

A number of previous studies have shown that Raman spectra from pathologically normal tissues near a tumor exhibit differences when compared to more distal tissues or non-tumor bearing subjects. These findings established a concept of cancer field effects (CFE) stating that the presence of malignancy in one anatomical region causes detectable and permanent changes to tissues elsewhere in the body (Slaughter *et al.*, 1953; Shen *et al.*, 2005). To test whether this CFE can be detectable by Raman spectroscopy, we measured 7 case and 3 control rats. Case animals received injection of 1M 13762MAT B-III into mammary gland. Control animals received 0.1ml of PBS. Raman measurements were performed on the overlying skin at the injection site, and at 5, 10, and 15mm from injection site towards contralateral nipple. Measurements were performed with a custom probe-based Raman system, 3 acquisitions per site, at day 0 and at 3 and 5 days post-injection. Raman data was processed to remove background artifact and tissue fluorescence. Partial least squares discriminant analysis (PLSDA) was performed to retrospectively assess capability to distinguish spectral changes in the two animal populations, using leave-one-out cross-validation. PLSDA scores show a clear separation of the case and control measurements at the furthest spatial measurement from the injection site (15mm) 3 days after injection of the tumor cells (Fig. 19 top) Loading plots of the latent variables comprising the discriminant show a number of Raman bands that are responsible for this segregation (Fig. 19 bottom) This analysis shows that Raman spectra can detect biochemical changes in distal skin after injection of tumor into mammary gland, which is hypothesized to be evidence of cancer field effect.

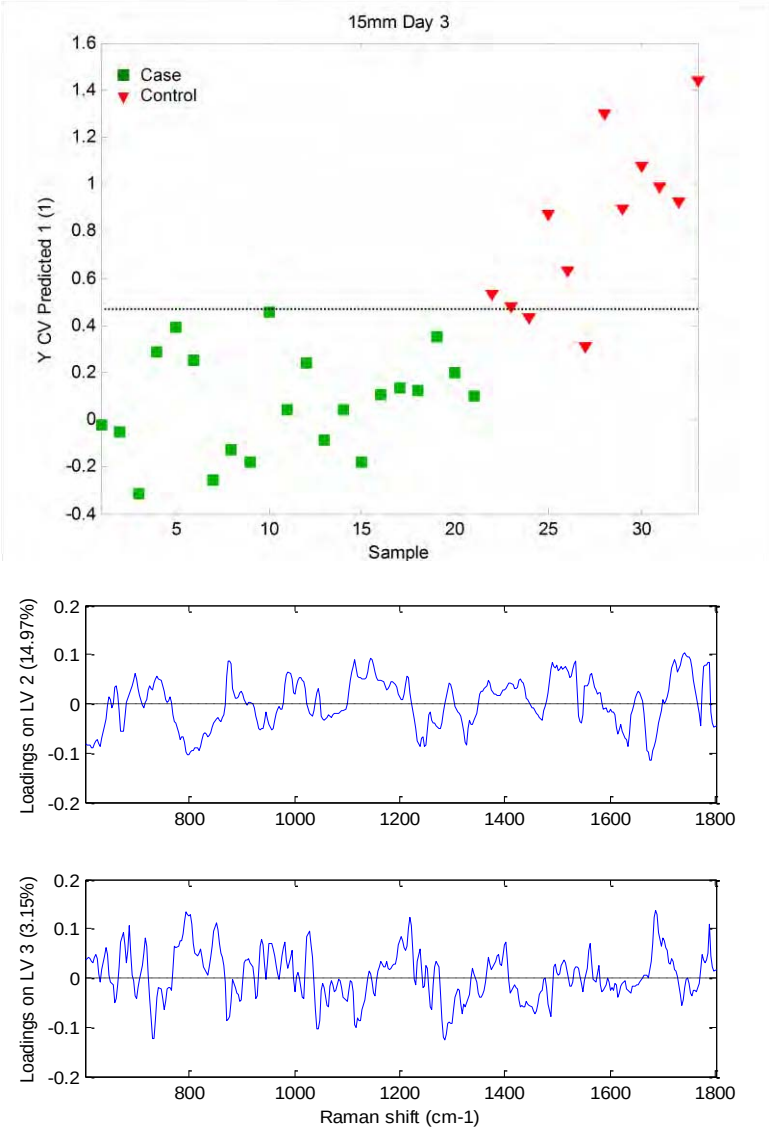

Fig. 19 The results from partial least squares discriminant analysis (PLSDA) on the two animal populations, using leave-one-out cross-validation. It shows a clear separation of the case and control measurements at the furthest spatial measurement from the injection site (15mm) 3 days after injection of the tumor cells (top). Loading plots of the latent variables comprising the discriminant show a number of Raman bands that are responsible for this segregation (bottom).

Key Research Accomplishments:

a. Found that oxy-, total hemoglobin concentration, and water content increase as tumors grow to certain size and then oxyhemoglobin decreases while deoxyhemoglobin increases, and water content continue to increase. The later changes tell us the formation of necrotic region in tumors.

b. Found that functional changes such as oxy-, deoxy-, total hemoglobin occurs even before tumor size change after chemotherapy. This implies that monitoring functional changes in tumors can predict the tumor response to treatment.

c. Observed that scattering information can be used to detect tumors from the surround tissues.

d. Found that MI system can be a useful tool to monitor the efficacy of vascular disrupting agents and compare efficacy among agents. CA4P and ECA caused decrease of oxyhemoglobin and increase of deoxyhemoglobin by blocking blood flow to tumors. CA4P worked better for the large tumors than small tumors in terms of both tumor growth delay and the amplitude of decrease in tumor oxygenation.

e. Compared the hemodynamic change of OHb and RHb in tumors treated by either cyclophosphamide or CA4P. Cyclophosphamide administration caused slight decreases of both OHb and RHb in tumors while normal breast showed an increase of OHb and stable RHb which may be from an acute inflammation response. The mechanism of CA4P is completely different from cyclophosphamide and therefore, we observed a decrease of OHb and an increase of RHb during 1hr post CA4P administration.

f. We employed a R3230AC mammary adenocarcinoma as another tumor line that received a cyclophosphamide treatment. R3230AC tumors partially responded to cyclophosphamide compared to the complete response shown from 13762 MAT B III tumors. R3230AC tumors also did not show flares of OHb, RHb, and THb during 1 week post cyclophosphamide treatment. However, water content continued to increase post chemotherapy. These results may support our hypothesis that therapy responding tumors show flares of OHb and/or RHb post treatment.

g. Raman spectroscopy was adapted to see whether we can see Raman signals change at the site far from the tumor cells injection site. Partial least square discriminant analysis scores show a clear separation of the case and control measurements at the furthest spatial measurement from the injection site (15mm) 3 days after injection of the tumor cells.

Reportable Outcomes

Presentations:

1) Jae G. Kim, Shigeto Ueda, Darren Roblyer, Austin Moy , Albert Cerussi, Wendy Tanamai, Amanda Durkin, Rita Mehta, David Hsiang, John Butler, Bruce Tromberg, "Early hemodynamic changes in breast tumors post chemotherapy: clinical and preclinical study", Department of Defense The Leading Innovation and Knowledge Sharing (LINKS) meeting, Chantilly, VA, Feb 16-17, 2010
2) Chad A. Lieber, Hubert E. Nethercott, Jae G. Kim, and Mustafa H. Kabeer, "Raman spectral detection of cancer field effects", presented at the 6th International SPEC conference, Manchester, UK June 26-July 1, 2010
3) Jae G. Kim, Tyler B. Rice, Shigeto Ueda, Edward Nelson, Albert Cerussi, Bruce J. Tromberg, "Changes in Endogenous Tissue Chromophores during Tumor Growth and post Cyclophosphamide Treatment on rat breast tumors", abstract submitted to SPIE photonics west, San Francisco, CA Jan 22-27, 2011

Research Opportunities:

One graduate student had research opportunities from this project. He was helping us to take images using MI system and to analyze the data. One visiting scholar from Japan became involved in this project. He is interested in monitoring neoadjuvant chemotherapy using a non invasive diffuse optical imaging, and helps us to perform immunohistological analysis.

Conclusions

From the work that I have conducted up-to-date, the following conclusions can be drawn.

As 13762 MAT B III tumors grow, increases of oxy-, total hemoglobin concentration, and water content were observed while deoxyhemoglobin decreased. However, oxyhemoglobin falls when tumors are bigger than $1cm^3$, but deoxyhemoglobin continues to increase resulting in the decrease of tumor oxygen saturation values. We found that functional changes such as oxy-, deoxy-, total hemoglobin occurs at day 2 post chemotherapy while tumor volume starts to decrease at day 3 post chemotherapy. This implies that monitoring functional changes in tumors can be a potential prognostic tool to early predict the tumor response to treatment. Vascular disrupting agents, combretastatin A4 phosphate (CA4P) and ECA, caused decrease of oxyhemoglobin and increase of deoxyhemoglobin by blocking blood flow to tumors. CA4P worked better for the large tumors than small tumors in terms of both tumor growth delay and the amplitude of decrease in tumor oxygenation. Compare to CA4P administration, cyclophosphamide administration caused slight decreases of both OHb and RHb in tumors while normal breast showed an increase of OHb and stable RHb which may be from an acute inflammation response. This result clearly demonstrated the mechanism differences between cyclophosphamide and CA4P. We employed a R3230AC mammary adenocarcinoma as another tumor line and applied a cyclophosphamide treatment. R3230AC tumors partially responded to cyclophosphamide compared to the complete response shown from 13762 MAT B III tumors. R3230AC tumors also did not show flares of OHb, RHb, and THb during 1 week post cyclophosphamide treatment. These results may support our hypothesis that therapy responding tumors show flares of OHb and/or RHb post treatment. We also adapted Raman spectroscopy to see whether we can see Raman signals change at the site far from the tumor cells injection site. Partial least square discriminant analysis scores show a clear separation of the case and control measurements at the furthest spatial measurement from the injection site (15mm) 3 days after injection of the tumor cells.

Overall, the monitoring of endogenous signals such as oxy-, deoxy-, total hemoglobin, water, and lipid using MI system can possibly predict tumor response to treatment earlier than monitoring tumor size change. Cyclophosphamide complete responding tumors exhibited flares of oxy-, deoxyhemoglobin while partial responding tumors did not. Vascular disrupting agents caused a significant decrease of tumor oxygenation within 1 hr post administration. It may be possible to detect tumor signals far away from tumors forming region using Raman spectroscopy.

It is very unfortunate that we could not accomplish our second year tasks. Our proposed model in task 3 was a dorsal skinfold window chamber model which inoculates tumor cells in the dorsal skin rather than in the breast of rats. However, we found that there are great differences in terms of tumor growth, metastasis, and therapy efficacy between ectopic and orthotopic tumor model. Therefore, we tried to adapt a mammary gland window model from Dr. Dewhirst's lab. However, we failed to reproduce their model in our lab and need to decide whether to follow our original dorsal skinfold window model or to have training from Dr. Dewhirst's lab.

We could not complete the proposed work in task 4. We performed a series of animal imaging under cisplatin treatment during our first year of this project. However, the spontaneous Brca1/p53 knockout mice tumor model normally takes 5 to 6 months to have tumors grow on the mice and we couldn't have enough animals to perform all the work in task 4. In addition, we were not able to obtain Tirapazamine drug. Instead of performing Task 3 and 4, we spent our efforts on 1) investigating oxy-, deoxyhemoglobin flares post chemotherapy using two different tumor lines, 2) monitoring and comparing the effects of vascular disrupting agents between small vs. large tumors in our animal model, 3) watching the Raman signal changes at the site far away from tumors. These new studies are summarized in this report.

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Appendices

Abstracts

1. Chad A. Lieber, Hubert E. Nethercott, Jae G. Kim, and Mustafa H. Kabeer, "Raman spectral detection of cancer field effects", presented at the 6th International SPEC conference, Manchester, UK June 26-July 1, 2010
2. Jae G. Kim, Tyler B. Rice, Shigeto Ueda, Edward Nelson, Albert Cerussi, Bruce J. Tromberg, "Changes in Endogenous Tissue Chromophores during Tumor Growth and post Cyclophosphamide Treatment on rat breast tumors", abstract submitted to SPIE photonics west, San Francisco, CA Jan 22-27, 2011

RAMAN SPECTRAL DETECTION OF CANCER FIELD EFFECTS

Chad A. Lieber,[1,2] Hubert E. Nethercott,[1] Jae G. Kim,[2] and Mustafa H. Kabeer[1]

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A number of previous studies have shown that Raman spectra from pathologically normal tissues near a tumor exhibit differences when compared to more distal tissues or non-tumor-bearing subjects. The sum of these collective findings have been hypothesized to be related to cancer field effects (CFE): the concept that the presence of malignancy in one anatomical region causes detectable and permanent changes to tissues elsewhere in the body. If true, this could facilitate the development of screening and diagnostic techniques for both preneoplastic tissues and distal malignancies via optical interrogation of readily accessible tissues. Towards this end, we have begun to directly evaluate the ability of Raman spectroscopy to detect CFE both in-vitro, using organotypic tissue raft cultures, and in-vivo, using animal models. Statistical analyses of these spectra reveal that the naive and tumor-associated tissues can be discriminated, though there is spatial and temporal dependence on this discriminant ability. These results encourage further study to more fully characterize the Raman capacity for detecting CFE as a possible tool for noninvasive screening for tumor presence.

2011 SPIE Meeting at San Francisco, CA Jan 22-27, 2011

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BO207 Optical Tomography and Spectroscopy of Tissue IX

Title

Changes in Endogenous Tissue Chromophores during Tumor Growth and post Cyclophosphamide Treatment on rat breast tumors.

Authors

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Abstract Text (250 words)

Previous clinical studies have shown that there are significant changes in optical signals after chemotherapy treatment within a few days. In this study, we have inoculated 1 million of 13762 MAT B III murine mammary tumor cells in the breast of Fisher 344 rats. When the tumor diameter reached ~1cm (~0.5cm^3), the rats were divided into the control (n=3) and treatment (n=5) group. Treatment group rats were treated with a single dose of cyclophosphamide (100 mg/kg, i.p.) while control group were administered with the same amount of saline. Tumor size and body weight were measured to monitor the therapeutic or side effects of cyclophosphamide treatment. A spatially modulated imaging system was employed to obtain oxy-, deoxy-, total hemoglobin concentration, tissue oxygen saturation, fat, and water of tumors during their growth and after cyclophosphamide administration. Oxy-, total hemoglobin concentration, and water increased as tumor grows while deoxyhemoglobin concentration initially decreased then increased as tumor grows. After cyclophosphamide treatment, tumors continued to grow two more days then regressed. However, oxy-, deoxy, and total hemoglobin concentrations reached their maximum at 1 day post cyclophosphamide treatment then gradually decreased along with tumor regression. For control tumors, we let them to grow ~2cm (~4.2cm^3) in diameter and watched optical signals changes. Oxy- and total hemoglobin increased until tumor volume is ~1.5cm^3 then decreased as tumors continue to grow while deoxyhemoglobin continued to increase. As a conclusion, we found that endogenous tissue chromophores respond to chemotherapy earlier than physical tumor size change which supports clinical reports.

Keywords

Breast Cancer, Diffuse Optical Imaging, Modulated Imaging, Cyclophosphamide, Chemotherapy